Current Status of the Host-Mediated L5178Y System for Detecting Chemical Mutagens

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Many chemicals which have been demonstrated to be mutagenic in a variety of organisms are either present in our environment or are used for human consumption. A variety of techniques have been developed for the detection of chemical mutagens. Some of these techniques have recently been reviewed and compiled (*1*). The important question, from the viewpoint of using these testing systems as a guide to human responsiveness, is one concerning the relevance of the results obtained from such tests to the actual or potential mutagenic hazard to man. We know too little to provide simple answers to the question, but a screening system utilizing the metabolism of potential mutagens or carcinogens by intact animals appears to be the most direct means of obtaining results that are as relevant as possible to man.

Fischer first developed a cell culture system for detection of mutagens using mouse leukemic L5178Y cells; this system was later combined with the metabolic capability of the animal host in the development of a host-mediated assay system in 1969 (*2*). This system seems to be very useful for detecting potential mutagens in man since it detects mutagenic effects of compounds in mammalian cells. These compounds are either directly mutagenic or not mutagenic themselves but are metabolized by the animal or man to form a mutagen. Although the basic genetic material is the same in all organisms and mutations are caused by the same types of DNA alteration, the mechanisms by which these alterations are induced and factors, such as repair system, transport across cellular membrances, structure of chromosomes, and metabolic activity, by which the processes are influenced, could be different in different organisms. Since we know very little about these mechanisms especially in higher organisms, results from mutagenesis systems employing a mammalian cell as the target cell may prove valuable in relating chemical mutagenicity data to human health hazards. We know that responsiveness of a compound to mutagenesis often varies greatly from one testing system to another.

One of the limitations of a host-mediated assay system is that the level of test compound must be nonlethal to the animal. It is established that most mutagens, including radiation, are highly effective only at concentrations that are lethal. This limitation, however, could be a pharmacological advantage because it reflects conditions of human exposure. It should be remembered that the question we are asking is not whether the compound might have mutagenic activity in some organism when exposed at any dosage,

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but whether the physiological doses of the compound in man are accompanied by any significant mutagenic or carcinogenic risk. The mutagenic action of the substance at a toxic level can be determined by *in vitro* assay with the same cell line. The comparison of this result with that of the host-mediated assay can then provide a clear understanding of the effect of host metabolism.

L5178Y is a fairly stable mammalian cell line with a near-diploid karyotype. These cells grow in suspension culture in large numbers, allowing statistically meaningful experiments. Sublines specially selected for mutagenesis studies possess a high level of infectivity in genetically compatible mice (BDF, DBA, AKR, etc.). Cells grow *in vivo* exponentially up to approximately 1×10^8 cells/ml in peritoneal fluid with a doubling time of approximately 14 hr. Cells obtained directly from the host are capable of reproducing with high cloning efficiency. The assay system was developed to detect the induction of forward point mutations as opposed to back mutations. The forward mutation system permits the detection of mutations at many genetic loci simultaneously, whereas the back mutation system usually permits the detection of mutations at only one genetic locus. In forward mutagenesis, the induced mutants can be of many different types revealing various point mutations. The back-mutation system is designed to detect a specific type of mutation (*3*). This combined with the well known fact that mutagenic specificity exists indicates that the forward mutation assay seems to be more suited as an initial screening test for potential mutagens. Various genetic markers are available for simultaneous detection of mutations at many different genetic loci in L5178Y cells and the biochemical consequences of the mutations have been elucidated in previous works (*4–6*).

Before any particular test system is used in the screening of a large number of chemicals for mutagenicity, it is important to determine whether the test system will in fact be effective in detecting a variety of known mutagens. The results would not only characterize the testing system for its sensitivity and specificity toward the various types of mutagens but also establish the optimum conditions and limitations of mutagenicity testing. As a part of our study on cancer therapeutic agents, we have examined a number of these agents for their mutagenic activity using the host-mediated assay system. Some of the results obtained in response to these drugs and certain known mutagens are presented here, and some of the problems connected with mutagenicity testing are also discussed.

In this study, we have chosen markers which confer resistance to three selective agents (thymidine, cytosine arabinoside, and methotrexate). Many different mutants with varying degrees of resistance comprise each type of mutant. Therefore, the mutant frequency (MF) changes with levels of selective agents employed. AKD_2F_1 hybrid mice weighing about 20 g each are used for this assay. In all the experiments presented here mice were inoculated intraperitoneally with 10^6 cells. Three days later drugs under investigation were administered by subcutaneous injection. Although a single dose was given by subcutaneous route in experiments discussed here, one advantage of this system is that by using lower inoculum of target cell it is possible to administer test substances daily by various routes for as long as two weeks. The drug dosages employed in this study were all nontoxic to the target cells in order to exclude the possibility of selection in favor of mutants. Three days after drug treatment the cells were recovered and mutant frequencies were determined using the soft-agar cloning method (*7*). Total cell number from each mouse was always determined in order to insure that the drug doses used were not toxic to the target cells. Optimum expression time for maximum mutant recovery was 2–3 days. Figures 1 and 2 and Tables 1–3 show examples of positive mutagenic response obtained from this study. Ethyl methanesulfonate (EMS) and 5-bromodeoxyuridine (BUdR) were used as positive controls. Because of low sensitivity, experiments frequently had to be repeated

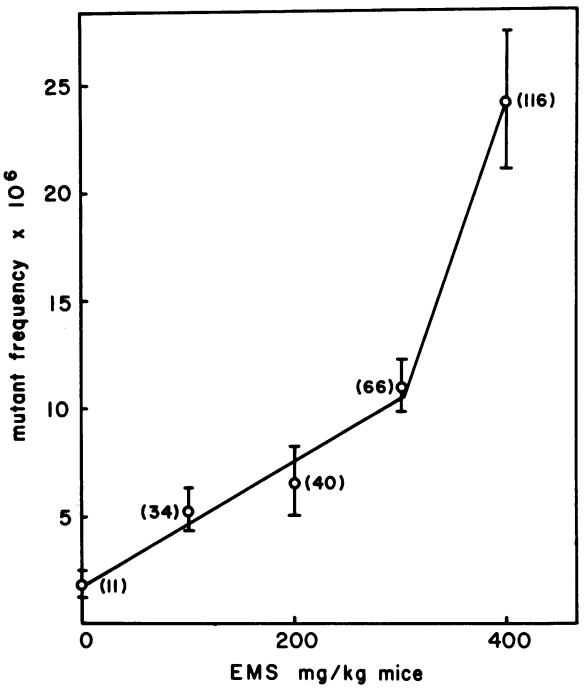

FIGURE 1. Induction of MTX-resistant mutants by EMS in the host-mediated assay. Each mouse was injected intraperitoneally with 10^6 cells. Three days after the inoculation, mice were injected subcutaneously with a single dose of EMS as indicated. Three days after the administration of EMS, cells were recovered from ascitic fluid and mutants were determined by the soft-agar cloning method using MTX as the selective agent. Numbers in parentheses indicate number of mutants recovered.

The standard error of the mean was calculated by using 15 samples from four experiments.

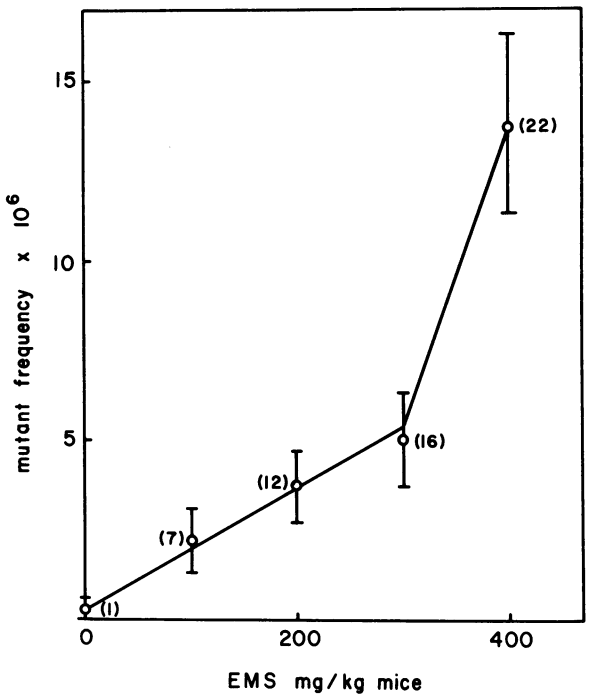

FIGURE 2. Induction of dTR-resistant mutants by EMS in the host-mediated assay. The assay procedure was exactly the same as that of Fig. 1, except dTR was used as the selective agent.

many times in order to obtain statistically meaningful results. Nevertheless, it was clear that this system responded well to some of the known mutagens. This system seems to respond to various alkylating agents (Figs. 1 and 2; Tables 2 and 3). Here the effect of host metabolism can also be seen. Both cytoxan and triethylene thiophosphoramide (thio TEPA) which gave positive response in this assay are known to require metabolic activation. Cytoxan was negative in our *in vitro* assay. It is interesting to note the high magnitude of response to BUdR and IUdR (Table 1). FUdR and a few other nucleoside analogs we tested were negative in this system. This system responded to at least one frameshift mutagen, hycanthone (Table 2) (*8*). We do not as yet know if this system responds only to certain types of frameshift mutagens. Proflavin, quinacrine, and ethidium bromide gave negative responses.

Before any significance concerning mutagenicity of a compound is discussed, especially of those which showed negative responses, it must be considered that all experi-

Table 1. Induction of MTX-Resistant Mutants by BUdR and IUdR in the host-mediated assay.

	Dose, mg/kg	MF × 10^6 [a]
BUdR	0	7.25 ± 2.0(4)
	150	81.5 ± 4.0(4)
	500	152.5 ± 30.2(4)
IUdR	0	9.25 ± 0.6(5)
	500	201 ± 25.0(5)

[a] Values in parentheses indicate number of determinations.

Table 2. Induction of mutation by TEM, thio-TEPA, and hycanthone.

	Dose, mg/kg	MF × 10^6 [a] MTX	MF × 10^6 [a] dTR
TEM	0	4.67 ± 1.27(7)	7.82 ± 2.87(4)
	0.5	22.9 ± 5.3(8)	34.0 ± 8.45(4)
Thio-TEPA	0	4.65 ± 1.12(9)	1.15 ± 0.32(9)
	7.5	20.9 ± 4.62(7)	50.25 ± 19.6(7)
Hycanthone	0	13.7 ± 2.1(4)	5.4 ± 2.22(4)
	100	36.2 ± 4.32(4)	19.4 ± 9.15(4)

[a] Values in parentheses indicate number of determinations.

Table 3. Induction of mutation by cyclophosphamide in the host-mediated assay.

Selective agent	MF × 10^6 [a] Control	MF × 10^6 [a] Cytoxan (100 mg/kg)
MTX	7.5 ± 1.4(16)	30.2 ± 4.2(8)
dTR	14.3 ± 2.9(16)	68.4 ± 13.2(8)
Ara-C	1.83 ± 0.67(15)	5.35 ± 1.6(7)

[a] Values in parentheses indicate number of determinations.

ments were carried out with acute, subtoxic administration of drugs. Another important consideration is the possibility that the increase in mutants represents selection by the test compounds. If the compounds were not inducing mutations but acting as selective agents then a large proportion of mutants would be recovered in the final population of treated cell samples. The fact that no toxicity to target cells was detected with the doses of drugs used in the assay (almost the same number of cells were harvested from the control and treated mice) suggests that there was no selection against the sensitive cells by the drugs. However, the possibility that mutants were selected against is not excluded, in which case the actual number of mutants induced would have been higher than that recovered, and the mutant frequencies given would have been underestimated. Reconstruction experiments must resolve this question, their purpose being to determine whether a selective advantage for mutants or the sensitive cell exists at the conditions of the standard assay and to further determine whether a testing compound has a selective lethal effect on mutants or sensitive cells. Our system is a forward mutagenesis in which many different types of mutants arise from mutations at more than one genetic locus. Therefore reconstruction experiments are very difficult to carry out. This may be one of the intrinsic disadvantages of a forward mutagenesis system. We have examined many mutants, either induced or derived spontaneously. A number of reconstruction experiments were performed using either isolated mutants or "representative" mutants which were a mixture of mutants recovered from any particular experiment. One such experiment is shown in Table 4. Results obtained from all the reconstruction experiments were entirely consistent with our previous interpretation that the increased number of mutants in the treated samples was induced by the mutagen. However, as seen in Table 4, for all the mutants tested there was an appreciable selection against mutants by the host animal, although their degree of selection was variable. This observation is somewhat surprising, because the *in vivo* growth rate of the mutants was similar to that of the sensitive cells when tested separately. One possible explanation is that sensitive cells affect the reproduction of mutant cells in culture and in mice. Although this host selective force hinders the quantitative measurement of mutagenesis in the host-mediated system, this is still a use-

ful system for the rapid screening of large numbers of potential mutagens and carcinogens. Positive controls such as EMS and BUdR have been used to estimate relative intensity of mutagenic compounds.

A great deal of additional information has been obtained from our mutant studies which was helpful to validate some important assumptions made for the host-mediated mutagenesis test system. This information includes: (1) the reproduction of mutant lines in the peritoneal cavity of mice has been found to be reproduceable and similar to the parent line; (2) various mutants obtained directly from the host grow in culture at a similar rate to sensitive cells (some mutants, however, grow a little slowly in the presence of the corresponding selective agents); (3) cloning efficiencies of mutants in the soft-agar method are similar to sensitive cells; and (4) recovery of mutants by the soft-agar method is unaffected by the presence of sensitive cells.

The most important assumption made in our system is that the phenotypic change to resistancy is caused by gene mutation. There are views which contradict this assumption (*9*). However, many biochemical studies of L5178Y mutants and other somatic mammalian culture cells, and studies of hybrid strains produced by fusing cells together with drug resistance are consistent with the interpretation that the phenotypic shifts are of genetic origin.

Table 4. Reconstruction of experiment with a MTX-resistant mutant (M_1) in the host-mediated assay.

	Inoculated			Recovered	
	No. of cells	MF	Treatment	No. of cells	MF
M_1	10^6	1	None	2.8×10^8	1.0
1% M_1	10^6	1×10^{-2}	None	2.5×10^8	0.12×10^{-2}
1% M_1	10^6	1×10^{-2}	EMS (250 mg/kg)	2.3×10^8	0.18×10^{-2}
1% M_1	10^6	1×10^{-2}	IUdR (500 mg/kg)	2.6×10^8	0.10×10^{-2}

One area for future improvement in the host-mediated L5178Y cell system would be the isolation of more sensitive sublines to mutagens, preferably repair enzyme-deficient mutants. This would not only improve sensitivity of the assay system, but would also provide valuable information on repair systems in mammalian cells. We are making an effort in this direction.

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